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Engineering of artificial microbial consortia of *Ralstonia eutropha* and *Bacillus subtilis* for poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) copolymer production from sugarcane sugar without precursor feeding

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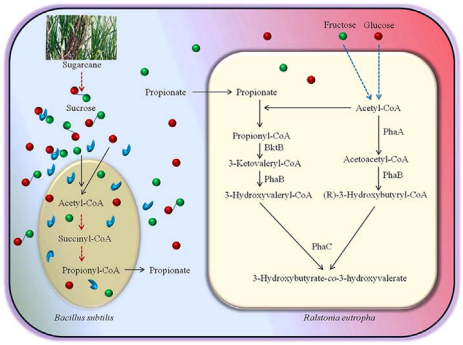
GRAPHICAL ABSTRACT



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ABSTRACT

Ralstonia eutropha is a well-known microbe reported for polyhydroxyalkonate (PHA) production, and unable to utilize sucrose as carbon source. Two strains, *Ralstonia eutropha* H16 and *Ralstonia eutropha* 5119 were co-cultured with sucrose hydrolyzing microbes (*Bacillus subtilis* and *Bacillus amyloliquefaciens*) for PHA production. Co-culture of *B. subtilis:R. eutropha* 5119 (BS:RE5) resulted in best PHA production (45% w/w dcw). Optimization of the PHA production process components through response surface resulted in sucrose: NH_4Cl:*B. subtilis*: *R. eutropha* (3.0:0.17:0.10:0.190). Along with the hydrolysis of sucrose, *B. subtilis* also ferments sugars into organic acid (propionic acid), which acts as a precursor for HV monomer unit. Microbial consortia of BS:RE5 when cultured in optimized media led to the production of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (P(3HB-*co*-3HV) with 66% w/w of dcw having 16 mol% HV fraction. This co-culture strategy overcomes the need for metabolic engineering of *R. eutropha* for sucrose utilization, and addition of precursor for copolymer production.

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1. Introduction

Polyhydroxyalkonates (PHA) has immense industrial potential, as it has many applications in tissue engineering, drug delivery, and packaging (Bhatia et al., 2016a; Lim et al., 2017; Panith et al., 2016). Various microbes are able to produce PHA under environmental and nutritional stress conditions, utilizing different carbon sources (Chen et al., 2017; Juengert et al., 2017; Vjayan and Vadivelu, 2017). Poly(3-hydroxybutyrate) (P(3HB)) is the most common type of PHA naturally accumulated by microbes, but has limited application, due to its rigidity, brittleness, and low thermal stability (Czerniecka-Kubicka et al., 2017; Obruca et al., 2016). Incorporation of various monomers in P(3HB) can improve its physical properties, so production of copolymer is a topic of current research interest. Copolymer P(3-hydroxybutyrate-*co*-3-hydroxyvalerate) P(3HB-*co*-3HV) is superior in properties as compared to P(3HB) due to its low melting point and level of crystallinity (Biernacki et al., 2017; Park et al., 2015). Different types of polymers can be produced by using a precursor feeding approach, or metabolic engineering of the microbe (Bhatia et al., 2015b; Srirangan et al., 2016). Acetic acid promotes 3HB production, propionate, and valerate plays a role in 3-hydroxyvalerate (3HV) synthesis in copolymer P(3HB-*co*-3HV), whereas butyrate acts as a precursor for hydroxyhexonate (HHx) unit in P(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (P(3HB-*co*-3HHx)) (Bhatia et al., 2015a; Jeon et al., 2014, 2017). The addition of all these external precursors in fermentation media adds to the cost.

Ralstonia eutropha is a well-known microbe for polyhydroxyalkonate production with higher productivity, but has limited carbon source utilization range (fructose, *N*-acetylglucosamine, gluconate, and a few organic acids etc.), and able to produce only P(3HB) (Bhatia et al., 2017b; Sichwart et al., 2011). Culture media components have greater influence on PHA production cost, so we wanted to extend *R. eutropha's* capability to utilize other cheaper carbon sources, and produce copolymer without addition of any precursor. Sucrose is a cheaper carbon source, and readily available in nature, as it comprises 90% of the total sugars in sugar cane (Zabed et al., 2014). However, wild type of *R. eutropha* is not able to utilize sucrose as a carbon source (Park et al., 2015). As an solution, sucrose hydrolysis into its free sugar (glucose and fructose) can be achieved by using invertase enzyme, but have limitation as it adds into cost when applied to large scale (Kehlbeck et al., 2014). Use of microbial consortia can be an option where one microbial strain can hydrolyze sucrose into free sugars, and these sugars further utilized by *R. eutropha* 5119 for PHA production. There are many examples of mixed and co-culture techniques to improve carbon utilization range and fermentation productivity (Bhatia et al., 2015c; Dwidar et al., 2013; Morgan-Sagastume et al., 2015). Different types of wild and engineered microbes can be co-cultured to construct natural, synthetic, or semisynthetic microbial consortia (Bhatia et al., 2017a,d). Wu *et al.* reported a microbial consortium for butanol production, where one partner *Bacillus cereus* scavenges the available oxygen, and provides a micro anaerobic environment for *Corynebacterium acetobutylicum* growth and butanol production (Wu et al., 2016). To increase carbon source utilization Xin *et al.* constructed a microbial consortium of *Kluyvera* sp. strain OM3 and *Clostridium* sp. strain BOH3 where the first strain hydrolyzes xylan and process resulted in increased production of butanol (Xin and He, 2013).

In this study, we have demonstrated a novel approach for copolymer P(3HB-*co*-3HV) production from sucrose without any precursor addition, by using a co-culture of PHA-accumulating *R. eutropha* 5119 and sucrose-hydrolyzing *Bacillus subtilis. B. subtilis* hydrolyzes sucrose into glucose and fructose, and also ferments these sugars into organic acids (acetic acid and propionic acid). *R. eutropha* 5119 utilize free sugars and organic acid for growth and P(3HB-*co*-3HV) production. Thus, this co-culture process presents a new approach for copolymer production from sucrose avoiding addition of costly precursors (propionic acid required for 3HV monomer unit), and an enzyme (invertase) required for sucrose hydrolysis.

2. Material and methods

2.1. Chemicals

All the chemicals for media were purchased from BD Difco laboratories (Becton-Dickinson, Franklin Lakes, NJ, USA). Reagents for GC, HPLC and GC–MS analysis (chloroform, methanol, and other derivatizing agent) and fatty acid methyl esters (FAMEs) standard (RM-5 and Rapeseed mix) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Microorganism, media, and culture condition

Various microorganisms used in this study, i.e. *Ralstonia eutropha* H16, *Ralstonia eutropha* 5119, *Bacillus subtilis,* and *Bacillus amyloliquefaciens,* were procured from the Korean Collection for Type Culture (KCTC). All strains were maintained on LB agar plate, and stored at 4 °C. Luria Bertani (LB) was used as media to prepare seed culture. Seed culture was prepared by inoculating a loop full of bacterial culture in 5 mL LB media, and incubating at 30 °C for overnight.

For PHA production, M9 minimal media, 5× (g/L, Na_2HPO_4 (33.9), KH_2PO_4 (15), NH_4Cl (5), NaCl (2.5) and sucrose (1%) was used. The microbes were cultured individually (0.1 mL seed culture), and for the consortia in combination of two, using 1:1 ratio seed culture, respectively, thereby keeping the final inoculum volume constant (0.1 mL). The microbes were cultured in 10 mL scale tubes having a total capacity of 25 mL at 30 °C, with agitation at 160 rpm for 72 h, after which an analysis was done for the PHA production and composition.

2.3. Analytical methods

Gas chromatography method with slight modification of the already reported method was used to analyze the PHA production and composition (Braunegg et al., 1978). On completion of growth, microbial culture was centrifuged at 10,000*g* to separate cells from the supernatant. Cell pellets were washed with deionized water two times, suspended in the minimum volume of deionized water, and subjected to lyophilization overnight. For methanolysis, approximately 10 mg of freeze-dried cells from each experiment were weighed, and placed in Teflon-stoppered glass vials, and 1 mL chloroform and 1 mL methanol/sulphuric acid (85: 15 v/v) were added to the vials. The vials were heated at 105 °C for two hours for methanolysis reaction. On completion of the reaction, the vials were cooled to room temperature, and 0.5 mL distilled water was added. The reaction mixture was vertexed for a few seconds, and allowed to separate into two layers. The lower layer was carefully collected, and added into an eppendorf tube containing Na_2SO_4. Samples were filtered by 0.22 mm Millex-GP syringe filter unit, and subjected to GC analysis. A 2 µL portion of the organic phase of these samples was then injected into a gas chromatograph (Agilent, Santa Clara, CA) equipped with a fused silica capillary column (Supelco SPB-5, 30 m × 0.32 mm, i.d. 0.25 µm film), with hydrogen as the carrier gas. The inlet of the gas chromatograph was maintained at 250 °C. The oven was held at 80 °C for 5 min, heated to 220 °C at 20 °C min^{-1}, and then held at 220 °C for 5 min. Peak detection was performed by a flame ionization detector, which was maintained at 300 °C.

An HPLC system equipped with a Bio-Rad Aminex HPX-87H column (Bio-Rad Co., Hercules, CA, USA) was used to analyze the components of various sugars and metabolite (Bhatia et al., 2017c,b). A mobile phase of 5 mM H_2SO_4 at a flow rate of 0.6 mL/min was used, and the column temperature was maintained at 50 °C.